Journal of the American Heart Association

ORIGINAL RESEARCH

Association of Inflammatory and Cardiovascular Proteomics Biomarkers With Indices of Heart Rate Variability in the General Population: KORA S4/FF4 Study

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BACKGROUND: We sought to investigate the association between circulating inflammatory and cardiovascular proteomics biomarkers and cardiac autonomic nervous dysfunction–sensitive heart rate variability indices.

METHODS: Using the population-based KORA (Cooperative Health Research in the Region of Augsburg) cohort, 233 proteomics biomarkers were quantified in baseline plasma samples of 1389 individuals using proximity extension assay technology. Five heart rate variability indices (Rényi entropy of the histogram with order [α] 4, total power of the density spectra, SD of word sequence, SD of the short-term normal-to-normal interval variability, compression entropy) were assessed at baseline in 982 individuals and in 407 individuals at baseline and at 14-year follow-up. Three unbiased multivariable selection models followed by linear or linear mixed-effects models with multiple testing correction were used to determine the association between proteomics biomarkers and heart rate variability indices.

RESULTS: C-C motif chemokine 23 was positively associated, while peptidoglycan recognition protein nd fibroblast growth factor 21 were negatively associated with Rényi entropy of the histogram with order (α) 4 cross-sectionally. Tumor necrosis factor–related activation-induced cytokine and growth/differentiation factor 15 were negatively associated with compression entropy cross-sectionally. Over time, interleukin-6 receptor subunit α and macrophage colony-stimulating factor were positively and negatively associated with total power of the density spectra, respectively. Additionally, myoglobin and agouti-related protein were positively and negatively associated with SD of the short-term normal-to-normal interval variability, respectively. Gastrotropin and agouti-related protein were positively and negatively associated with compression entropy, respectively.

CONCLUSIONS: This study identified novel circulating proteins associated with heart rate variability indices. These proteins could improve our understanding of the pathophysiology underlying cardiac autonomic nervous dysfunction.

Key Words: agouti-related protein ■ cardiac autonomic nervous dysfunction ■ growth/differentiation factor 15 ■ heart rate variability ■ macrophage colony-stimulating factor, gastrotropin ■ myoglobin ■ proteomics biomarkers

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Part of this work was presented at the German Diabetes Congress, May 28–31, 2025, in Berlin, Germany.

This manuscript was sent to Ajay K. Gupta, MD, MSc, PhD, FRCP, FESC, Senior Associate Editor, for review by expert referees, editorial decision, and final disposition.

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.125.042144

For Sources of Funding and Disclosures, see page 17.

JAHA is available at: www.ahajournals.org/journal/jaha

RESEARCH PERSPECTIVE

What Is New?

- This epidemiological study observed that 10 novel circulating proteins are associated with cardiac autonomic nervous dysfunction–sensitive heart rate variability indices.

What Question Should Be Addressed Next?

- Future studies using larger study samples should profile these novel proteins and their associated cardiac autonomic nervous dysfunction–sensitive heart rate variability indices at multiple time points to investigate the temporal variation of these proteins and indices, the impact of intraindividual variation on the association between the proteins and the indices and the relationship between their trajectories.

Nonstandard Abbreviations and Acronyms

AGRP	agouti-related protein
CAND	cardiac autonomic nervous dysfunction
CCL23	C-C motif chemokine 23
CE	compression entropy
CSF1	macrophage colony-stimulating factor 1
DDA	direction dependence analysis
FGF21	fibroblast growth factor 21
GDF15	growth/differentiation factor 15
HbA_{1c}	hemoglobin A1c
HRV	heart rate variability
IL6RA	interleukin-6 receptor subunit α
KORA	Cooperative Health Research in the Region of Augsburg
MUVR	multivariable modeling with unbiased variable selection methodsNGTnormal glucose tolerance
PGLYRP1	peptidoglycan recognition protein 1
PLS	partial least squares regression
Rényi4	Rényi entropy of the histogram with order (α) 4
RF	random forest regression
RMSE	root mean square error
SDSA	SD of the short-term normal-to-normal interval variability
SDWS	SD of word sequence
T2D	type 2 diabetes
TP	total power of the density spectra
TRANCE	tumor necrosis factor–related activation-induced cytokine

Type 2 diabetes (T2D) accounts for >90% of all diabetes globally.[1] Cardiac autonomic nervous dysfunction (CAND), a dysfunction of sympathetic or parasympathetic activity or regulation, is a prevalent, serious, and often overlooked diabetes-related complication.[2–6] Important sequelae of CAND are increased risk of major cardiovascular events and death.[2,6] Heart rate variability (HRV) alterations are the hallmark of CAND.[2] Consequently, HRV indices have become the most popular and widely used tool for the identification of CAND.[6–8] In the population-based KORA (Cooperative Health Research in the Region of Augsburg) study, we previously reported that a combination of 4 short-term HRV indices selected from multiple classes of linear and nonlinear HRV dynamics (ie, Rényi entropy of the histogram with order [α] 4 [Rényi4], total power of the density spectra [TP], SD of word sequence [SDWS], and SD of the short-term normal-to-normal interval variability [SDSA]) resulted in the most sensitive estimate of CAND prevalence in the general population.[9] These CAND-sensitive HRV indices (henceforth HRV indices) could provide a deeper understanding of CAND.

Risk factors for CAND include age,[4,5] obesity,[3–5,10] physical inactivity,[5] smoking,[5] dyslipidemia,[3–5] and hypertension.[2–5,10] Interestingly, dysglycemia,[3,5,10] known diabetes duration,[7] impaired kidney function,[5] retinopathy,[2] other neuropathies,[2,11] medications,[7,9] but also genetic predisposition.[5] Indeed, a multifactorial intervention of lifestyle changes and targeting glucose and cardiovascular disease (CVD) risk factors is recommended for the prevention of CAND.[7] Of note, CAND is more than a diabetes-related complication as it is also prevalent in individuals with prediabetes and in advanced age.[5,7,10] This underscores the pressing need to further explore the risk factors and biomarkers of CAND. Population-based epidemiological studies with glucose tolerance status of individuals in advanced age could be an excellent resource to address this need.

The pathophysiological underpinnings of CAND are complex.[11] Nonetheless, its integral molecular mechanisms involve insulin resistance,[5] dysregulated inflammation,[5,12] and oxidative stress.[12] Indeed, targeting some biomarkers of inflammation and endothelial function has been suggested to be promising for the treatment of CAND.[12] Expectedly, some cardiovascular and inflammatory biomarkers, CRP (C-reactive protein)[13] and adiponectin,[14] were found to be associated with CAND in clinic-based epidemiological studies, while CRP,[15] interleukin-6,[15] interleukin-18,[16] interleukin-1 receptor antagonist,[15] and adiponectin[16] have been linked to CAND in population-based cohorts. However, only a few of these associations remained when classical cardiometabolic risk factors were taken into account, suggesting that most are not independent biomarkers

of CAND and its HRV-related indices. Additionally, the selected biomarkers of previous studies might be unable to capture important aspects of the apparently broad and complex pathophysiological underpinnings of CAND. Indeed, population-based studies with optimized targeted quantification of an array of well-defined set of proteomics biomarkers could advance this investigation. Furthermore, it is unknown whether these proteomics biomarkers would be relevant for CAND beyond the commonly assessed inflammatory biomarkers. While it seems intuitive that alterations in proteomics biomarkers influence these indices, the potential bidirectional relationship between inflammation and CAND[17] suggests that the relationship between these biomarkers and HRV indices needs to be properly disentangled.

Hence, this large population-based epidemiological study sought to investigate the independent associations between plasma circulating proteomics biomarkers and HRV indices cross-sectionally and over time.

METHODS

The data are subject to national data protection laws. Therefore, data cannot be made freely available in a public repository. However, data can be requested through an individual project agreement with KORA. To obtain permission to use KORA data under the terms of a project agreement, please use the digital tool KORA. PASST (https://epi.helmholtz-muenchen.de/).

Study Population and Design

The current study is based on data from the population-based KORA S4 cohort (1999–2001) and its 14-year follow-up, KORA FF4 (2013–2014). In 1999, study participants were recruited from the region of Augsburg (Germany) using random sampling and random selection of 16 towns and villages from 70 communities. Sex- and age-stratified sampling was done for each community. Four of the strata comprised men and women aged 55 to 74 years. Participants provided biosamples that included fasting blood samples. Venipuncture was performed on participants in a sitting position. The blood samples were stored at –196 °C in liquid nitrogen until plasma proteomics analysis in 2019 to 2020. Medical history was obtained through a structured interview, and various medical assessments such as ECGs were also performed. Details of the design of the KORA S4/F4/FF4 cohort and assessments have been previously described.[9,18,19] All investigations were conducted in accordance with the Declaration of Helsinki, and all participants provided written informed consent. The ethics committee of the Bavarian Chamber of Physicians, Munich approved all study protocols.

This present analysis is based on KORA study participants at baseline (S4) comprising 1653 individuals, aged 55 to 74 years. We sequentially excluded 88 individuals who had missing data on any of the exposure variables (previously analyzed 233 proteomics biomarkers[20]) at S4, 49 individuals with missing data on any of the outcome variables (5 selected HRV indices) at S4, and 127 individuals with unclear glucose tolerance status due to missing oral glucose tolerance test data. This resulted in 1389 eligible S4 individuals. There were no individuals with type 1 diabetes. Of this study population, there were 407 with complete data on the 5 HRV indices at follow-up (FF4). Hence, the overall 1389 study population comprised 982 nonoverlapping individuals with 1-time assessed outcome variables (HRV indices) at S4 and 407 individuals with 2 repeatedly assessed HRV indices at baseline and follow-up (FF4). These nonoverlapping analytical study samples (henceforth referred to as S4 and S4-FF4 study samples, respectively) were used to determine the associations of proteomics biomarkers with HRV indices cross-sectionally and over time, respectively. Findings from both study samples are complementary, providing internal generalization to the overall study population. Figure 1 shows the flowchart of the study population.

Measurement of the Exposure: Proteomics Biomarkers

CVD- and inflammation-related protein biomarkers were measured in baseline plasma samples using the targeted proximity extension assay technology developed by Olink (Olink Proteomics, Uppsala, Sweden) with the 3 panels Olink Multiplex CVDII, CVDIII, and Inflammation. These panels were designed for broad inflammation- and CVD-related research questions. While they are not specific to HRV- or CAND-related hypotheses, inflammation is generally considered as an important driver of CAND. To avoid batch effects, samples were randomized across plates. Each plate included interplate controls, which were used to adjust for any plate difference.[21] The Olink platform provides protein abundances as protein expression values, which are similar to $\log_2$-normalized concentrations. Details of the proximity extension assay method are reported elsewhere.[20,21] For this cohort's exposure variable, we considered 233 previously analyzed proteomics biomarkers.[20] These 233 biomarkers comprised 85, 81, and 67 biomarkers from the CVDII, CVDIII, and Inflammation panels, respectively.

Assessment of Covariates: Sociodemographic, Anthropometric, and Lifestyle Factors and Other Biomarkers

Information on age, sex, education, smoking habits, alcohol consumption, physical activity, and medical history were collected by personal interviews conducted

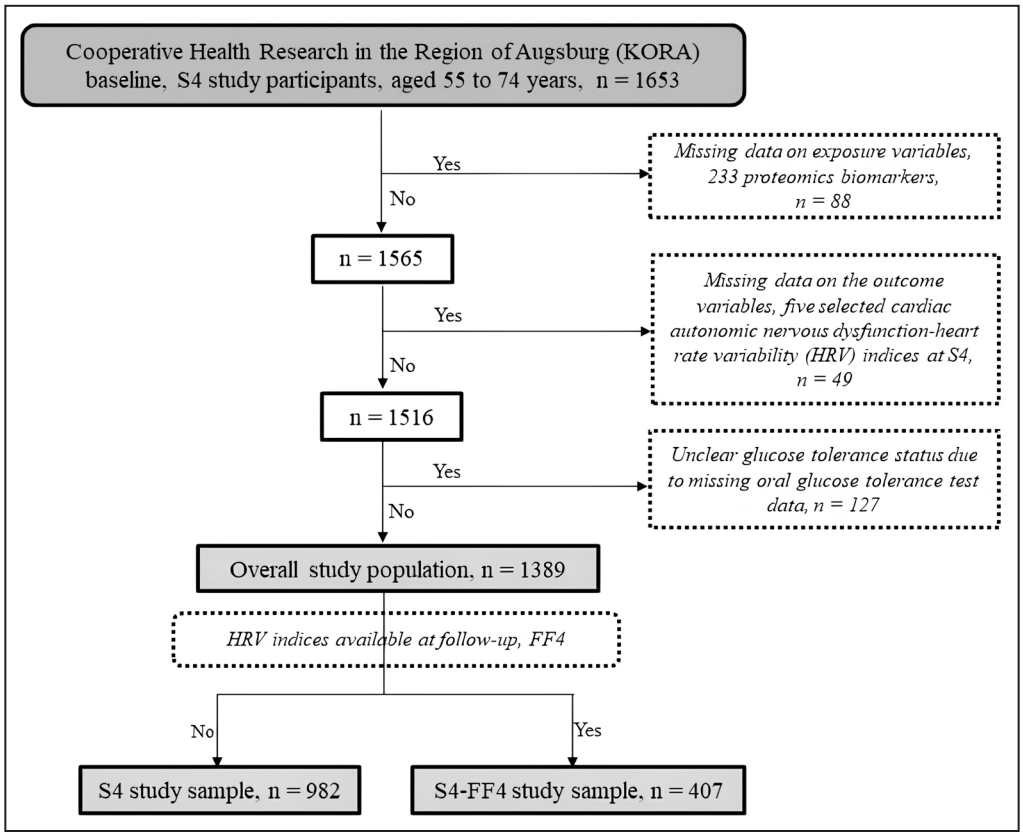

Figure 1. Flowchart of the study population.

by experienced medical staff. Educational attainment was recorded as completed years of schooling. Height, weight, waist circumference, and systolic and diastolic blood pressure were measured at the study visit on the basis of standard protocols, as described elsewhere.[9,18,19] Body mass index (BMI [kg/m^2]) was calculated from weight and height. Smoking habits and alcohol consumption were self-reported. Smoking status was categorized as nonsmokers, former smokers, and current (regular and irregular) smokers. Alcohol consumption was based on reported intake of beer, wine, and liquor on 1 weekday and the weekend. It was expressed in g/d. Participants estimated the duration and frequency of their weekly exercise across summer or winter. They were categorized as either physically active (≥1 hour sports/wk) or inactive. Blood pressure was measured 3 times at the right arm after a 5-minute resting period. The mean of the second and third measurements was used for analyses. Medication use was defined using Anatomical Therapeutic Chemical Classification System codes. From baseline plasma samples, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides were measured by enzymatic methods.[22] Hemoglobin A_{1c} (HbA_{1c}) was measured by immune turbidimetric assays.[23] An oral glucose tolerance test was performed using standard procedure on those without previously known T2D. Individuals were categorized into six glucose tolerance groups of normal glucose tolerance (NGT), isolated impaired fasting glucose, isolated impaired glucose tolerance, combined isolated impaired fasting glucose–isolated impaired glucose tolerance, newly detected T2D and previously known T2D as previously described by Ziegler et al.[9]

In addition to commonly assessed biomarkers, leukocyte count was quantified with the Coulter STKS Hematology Analyzer (Block Scientific, New York, NY), and CRP was quantified using a high-sensitivity latex-enhanced nephelometric assay on a BN II System analyzer (Dade Behring, Marburg, Germany), while serum amyloid A and fibrinogen were determined by immunonephelometry.[24] Adiponectin was determined with the human adiponectin RIA from Linco Research (St. Charles, MO).[25]

Assessment of Outcomes: HRV Indices

The assessment of HRV indices has been previously described.[9] Briefly, ECGs (lead II and lead V2 simultaneously) were recorded in the supine resting position over a period of 5 minutes (sample frequency, 500 Hz). Time series of heart rate (tachograms) consisting of beat-to-beat intervals were extracted from the 5-minute ECG recordings. Individuals with atrial

fibrillation or flutter, left and right bundle-branch block, second- and third-degree atrioventricular block or sinoatrial block, multiple supraventricular or ventricular extrasystoles, pacemaker therapy, and treatment with class I antiarrhythmics were excluded. A total of 120 HRV variables (time domain [statistical and geometric analysis], 15 indices; frequency domain [spectral analysis], 15 indices; nonlinear dynamics, 90 indices using 8 different methods) were determined by applying linear and nonlinear HRV analysis methods to the filtered tachograms. Calculations of the indices were performed using in-house software.

The present analysis considered the 4 indices from 4 different HRV domains: Rényi4 (bit), TP (ms^2), SDWS, and SDSA (ms), which were previously reported to be optimal for estimating the prevalence of CAND.[9] We included 1 additional HRV index, compression entropy (CE), that showed promising association with CAND.[9] Overall, we analyzed 5 HRV indices (Rényi4, TP, SDWS, SDSA, and CE) for both study samples. The clinical relevance of these indices is provided in Data S1.

Statistical Analysis

Descriptive Analysis

Continuous and categorical basic characteristics (covariates) of the overall study population and each study sample, were summarized as median (interquartile range), and count (percentage), respectively. Comparison of the continuous and categorical covariates between the S4 (n=982) and S4-FF4 (n=407) study samples were tested with the Kruskal–Wallis rank-sum test and Pearson's χ^2 test, respectively. Kruskal–Wallis rank-sum test was done to compare the 2 groups, S4, and S4-FF4 study samples. Therefore, no post hoc test was needed.

Multivariable Modeling of the Association Between Proteomics Biomarkers and HRV Indices

Figure 2 displays the statistical analytical plan. We partitioned the S4 into 3 (training, validation, and testing) nonoverlapping data sets using 50:25:25% split[26] and S4-FF4 into 2 (training and testing) nonoverlapping data sets, using 80:20% split.[27] These partitions were stratified on 6 glucose tolerance groups (NGT, isolated impaired fasting glucose, isolated impaired glucose tolerance, combined isolated impaired fasting glucose–isolated impaired glucose tolerance, newly detected T2D and previously known T2D), which were previously used to estimate CAND prevalence in this study population.[9] Thus, the S4 comprised 490 training, 246 validation and 246 testing data sets, while the S4-FF4 comprised 325 training and 82 testing data sets. The S4 and S4-FF4 training data sets were used for predictor variable selection. The S4 validation data set was used for inferential analysis, and the S4 testing data set was used for prediction modeling. The S4-FF4 testing data set was used for inferential analysis and prediction.

S4 Study Sample

The S4 training (variable selection) data set was used to identify important predictor variables (exposure variables and covariates) of each of the 5 HRV indices (Rényi4, TP, SDWS, SDSA, and CE). We used 3 multivariable modeling with unbiased variable selection methods (MUVR), partial least squares (MUVR-PLS), random forest (MUVR-RF) and elastic net (MUVR-EN) regression.[28,29] Further details are provided in Data S1. The MUVR algorithm returns 3 different consensus models, minimal-optimal (strongest predictors), "mid" and all-relevant (strongest and entirely redundant predictors). We chose predictor variables from the "mid" consensus model, which is a trade-off between the minimal-optimal and the all-relevant models. Predictor variables shared by all the 3 methods, MUVR-PLS, MUVR-RF, and MUVR-EN were considered as robust predictor variables. Since glucose tolerance status is central to this investigation, the inclusion of any glucose tolerance group in the robust predictor variables has a relaxed criterion of selection by only MUVR-RF, owing to the ability of RF to uncover complex and important interactions between variables[30] (details in Figure 2).

Each HRV index assessed at baseline was separately regressed on the predictor variables, measured at baseline. The exposure variables were the protein expression values of 233 proteomics biomarkers. We performed a priori selection of covariates, and the final covariates were the minimal sufficient adjustment set of confounders estimating the direct effect of the proteomics biomarkers on the HRV from the directed acyclic graph (Figure S1). The general direction of proteomics biomarkers–confounder association was based on prior knowledge or literature on the well-known proteins within the 233 proteomics biomarkers. The directed acyclic graph–selected covariates were age, sex (men; reference: women), BMI, waist circumference, smoking status (smokers, ex-smokers; reference: nonsmokers), alcohol intake, educational attainment, physical activity (active; reference: inactive), high-density lipoprotein cholesterol, low-density lipoprotein cholesterol cholesterol, triglycerides, systolic and diastolic blood pressure, medications (selected medications with possible influence on HRV by Ziegler et al[9]: β blockers, angiotensin-converting enzyme inhibitors, angiotensin antagonists, calcium antagonists, and others: glucose-lowering drugs, diuretics, statins, NSAIDs; reference: nonusers), uric acid, creatinine, CRP, leukocyte, adiponectin, albumin, fibrinogen, and serum amyloid A, HbA_{1c}, glucose tolerance groups (isolated impaired fasting glucose,

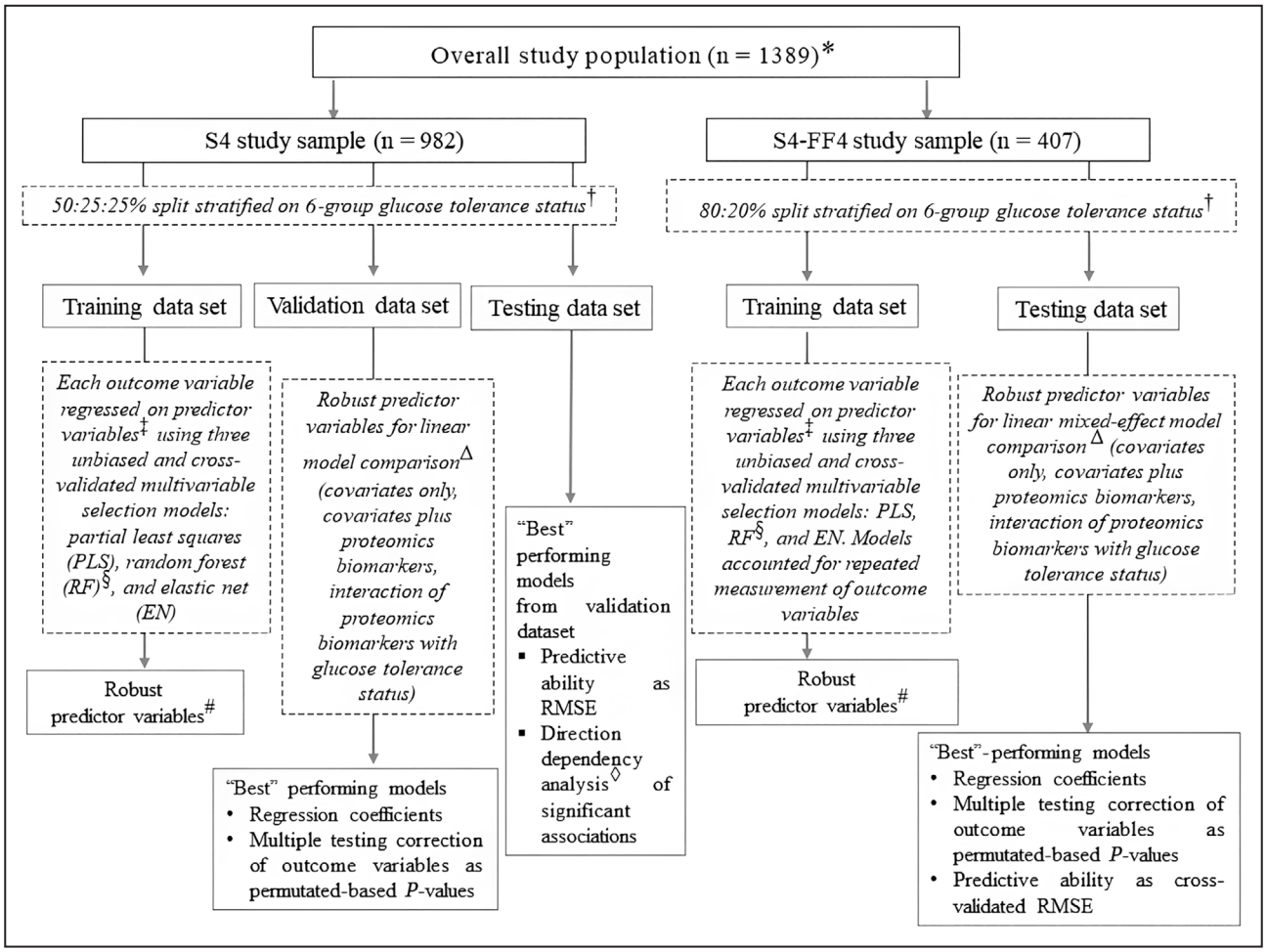

Figure 2. Statistical analytical plan.

*Nonmissing on exposure variables (233 proteomics biomarkers), outcome variables (5 CAND–HRV indices), and glucose tolerance status. †Normal glucose tolerance, (i-IFG, i-IGT, combined IFG–IGT, newly detected T2D, and known T2D. ‡Predictor variables: 233 proteomics biomarkers and directed acyclic graph-selected covariates. §Relaxed inclusion of glucose tolerance status: selection by only RF. #Predictor variables shared by all three methods. ΔDependent on the set of robust predictor variables. ◊Compares putatively correct and reverse causal order; training data sets: variable selection data sets. Validation and testing data sets: model fitting data sets. CAND, cardiac autonomic nervous dysfunction; HRV, heart rate variability; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; i-IFG, isolated impaired fasting glucose; i-IGT, isolated impaired glucose tolerance; RF, random forest regression; RMSE, root mean square error; and T2D, type 2 diabetes.

isolated impaired glucose tolerance, combined isolated impaired fasting glucose–isolated impaired glucose tolerance, newly detected T2D and previously known T2D; reference: NGT) and known T2D duration. Plausible values of missing covariates were single-value imputed using the nonparametric multivariate imputation by the chained RF. All continuous predictor variables were further *Z* score standardized (mean, 0±1).

Using linear models, the robust predictor variables were validated on the S4 validation (first model fitting) data set. Depending on the set of robust predictor variables, we compared basic (covariates only: reference), full (robust predictor variables) and complex-full models. The complex-full model would be the full model with 2-way multiplicative interaction of each proteomics biomarker with any glucose tolerance group recovered as a robust predictor variable. Models with the highest overall performance scores (mean of normalized performance metrics comprising the coefficient of determination, root mean squared error [RMSE], residual SD, Akaike information criterion, and Bayesian information criterion) were chosen as the "best"-performing models. In case of equal performance scores, models with fewer predictor variables were selected as the best-performing model. No model comparison was performed for HRV indices in which the robust predictor variables were only proteomics biomarkers. These proteomics biomarkers-only models were considered as the best-performing models. We estimated β and 95% CI of the best-performing models. To further account for the multiple testing of the correlated HRV indices, the highly statistical powered permutated *P* values[31]

were computed using 5000 permutations (Data S1). We considered significant proteomics biomarkers as those with permutated $P<0.05$. Furthermore, we performed bias analysis of the β by determining the robustness of inference to replacement and impact threshold of a confounding variable[32] (Data S1). The best-performing models' predictive ability with the RMSE was evaluated on the S4 testing (second model fitting) data set.

Finally, considering the cross-sectional nature of the S4, we used directional dependency analysis (DDA)[33] to empirically confirm whether the a priori (putatively correct) causal order (proteomics biomarkers⟶HRV indices) is more likely to reflect the correct causal flow over the alternative (reverse) causal order (HRV indices⟶proteomics biomarkers). We tested only the statistically significant proteomic biomarkers using the S4 testing (second model fitting) data set. The decision of explanatory superiority was based on the standard and studentized (robust) Breusch–Pagan homoscedasticity tests and bootstrap Hilbert–Schmidt independence criterion test with 1000 resamples (Figure 2).

S4-FF4 Study Sample

To recover the robust predictor variables of the S4-FF4, the aforementioned multivariable selection steps were performed on the training (variable selection) data set. The repeatedly measured HRV indices were regressed on the predictor variables measured at baseline, using MUVR-PLS, MUVR-RF, and MUVR-EN (Figure 2).

Next, we fitted the robust predictor variables, performed model comparison, and model inference on the testing (model fitting) data set using linear mixed-effects (random-effects) models. The outcome variables were the repeatedly measured HRV indices. All robust predictor variables were modeled as fixed effects and a random effect (intercept) was specified for every individual. The β indicates the effect of the robust predictor variables on the average HRV indices over time. Bias analysis was performed on the significant proteomics biomarkers of the best-performing models. The predictive ability of the best-performing models were evaluated on the same testing (model fitting) data set as leave-one-out cross-validated RMSE (test RMSE) (Figure 2). No DDA was performed in the S4-FF4 because its longitudinal design with subsequently measured HRV indices at follow-up (temporality) indicates an established causal order (proteomics biomarkers⟶HRV indices).

Independence of Proteomics Biomarkers, Bivariable Associations, Statistical Power, and Individual Power Components

Before the multivariable regression modeling, we checked the dependency among the 3 panels of proteomics biomarkers as well as bivariable associations of predictor variables. The association between continuous variables was tested with Spearman correlation test, while difference across the groups of categorical variables was tested with the Kruskal–Wallis test. Furthermore, we estimated the statistical power of the generalized linear model of the partitioned data sets. Details are provided in Data S1. In secondary analysis, we examined the association of the proteomics biomarkers with individual power components, in the very-low-frequency, low-frequency, and high-frequency range, using the same analytical steps as in the main analysis.

All statistical analyses were performed using R version 4.3.3. The R packages were "MUVR2" for multivariable selection, "performance" for model comparison, "lmPerm" for permutation of linear models, "dHSIC" for DDA, "permutes" for permutation of linear mixed models, and "konfound" for bias analysis; "caret" for predictive ability (RMSE); and "pwr" for a priori statistical power analysis. We considered $P<0.05$ as statistically significant.

RESULTS

Descriptive Analysis

Table 1 summarizes the basic characteristics of the overall study population (n=1389) and the S4 (n=982) and S4-FF4 (n=407). The overall study population had 52% men, a median age of 64 years, and a median BMI of 28 kg/m^2; 42% were physically active, 14% were current smokers, and 60% had NGT. The S4 had 54% men, age 65 years, and BMI of 28 kg/m^2; 40% were physically active, 15% were current smokers, and 56% had NGT, while the S4-FF4 had 48% men, age 61 years, and BMI of 27 kg/m^2; 48% were physically active, 12% were current smokers, and 68% had NGT. The median follow-up time of the S4-FF4 was 14 years. Basic characteristics such as age, BMI, and smoking status were significantly different between the S4 and S4-FF4. Tables S1 and S2 provide the data for all proteomics biomarkers and HRV indices, respectively, for the overall study sample and the S4 and S4-FF4 populations.

Multivariable Modeling of the Association Between Proteomics Biomarkers and HRV Indices

Association Between Proteomics Biomarkers and HRV Indices in S4 Study Sample

There were 16 (12 proteomics biomarkers and 4 covariates), 6 (all proteomics biomarkers), 10 (9 proteomics biomarkers and 1 covariate), 7 (all proteomics biomarkers) and 10 (9 proteomics biomarkers and one covariate)

Table 1. Basic Characteristics of the Study Population

	Overall (n=1389)	S4 study sample (n=982)	S4-FF4 study sample (n=407)	*P* value*
Age, y	64 (59–69)	65 (61–70)	61 (58–65)	<0.001
Sex, male	725 (52.2)	528 (53.8)	197 (48.4)	0.068
Body mass index, kg/m^2	28.2 (25.7–30.9)	28.5 (25.9–31.3)	27.4 (25.4–30.0)	<0.001
Waist circumference, cm	96.1 (88.6–103.2)	97.1 (90–104.5)	93.7 (85.9–101.0)	<0.001
Educational attainment, y	10 (10–12)	10 (10–12)	10 (9–12)	0. 100
Alcohol consumption, g/d	7 (0–22.9)	6.6 (0– 22.9)	8.6 (0.9–22.7)	0.626
Smoking status, smokers	192 (13.8)	144 (14.7)	48 (11.8)	0.020
Physical activity, inactive	796 (57.6)	586 (60)	210 (51.7)	0.005
Systolic blood pressure, mm Hg	135 (122.5–148)	137 (123.5–149.5)	131 (119–145)	<0.001
Diastolic blood pressure, mm Hg	79.5 (73–86.5)	79.5 (73.0–87.0)	80 (73.5–86.0)	0.853
Hemoglobin A_{1c}, mmol/mol	38 (36–41)	39 (36–41)	38 (36–41)	0.237
High-density lipoprotein cholesterol, mmol/L	1.4 (1.2–1.7)	1.4 (1.2–1.7)	1.5 (1.2–1.8)	0.037
Low-density lipoprotein cholesterol, mmol/L	3.9 (3.3–4.6)	3.9 (3.3–4.6)	3.9 (3.2–4.6)	0.616
Triglycerides, mmol/L	1.4 (1.0–1.9)	1.4 (1.0–2.0)	1.3 (0.9–1.8)	0.003
Albumin, g/L	38.2 (35.8–40.7)	38.1 (35.7–40.6)	38.5 (36.2–40.9)	0.049
Fibrinogen, g/L	2.8 (2.5–3.3)	2.9 (2.5–3.3)	2.7 (2.4–3.2)	0.005
High sensitivity C-reactive protein, mg/L	1.7 (0.9–3.5)	1.9 (0.9–3.8)	1.5 (0.8–2.9)	0.001
Serum amyloid A, mg/L	3.6 (2.4–6.1)	3.7 (2.4–6.4)	3.4 (2.3–5.5)	0.113
Leukocyte count, /nL	5.9 (5.1–7.0)	8.8 (6.2–12.2)	8.4 (5.6–1.8)	0.058
Serum adiponectin, µg/mL	8.7 (6.0, 12.2)	6 (5.0–7.0)	5.7 (5.0, 6.7)	0.001
Uric acid, µmol/L	329.2 (278.6–391.7)	334.3 (281.6–397.6)	318.5 (270.2–373.5)	<0.001
Creatinine, µmol/L	75.2 (66.3–85.8)	75.2 (66.3–85.8)	74.3 (65.4–84.0)	0.305
Use of angiotensin antagonists	46 (3.3)	36 (3.7)	10 (2.5)	0.249
Use of angiotensin-converting enzyme inhibitors	178 (12.8)	150 (15.3)	28 (6.9)	<0.001
Use of calcium antagonists	149 (10.7)	124 (12.7)	25 (6.1)	<0.001
Use of β blockers	294 (21.2)	232 (23.7)	62 (15.2)	<0.001
Use of diuretics	230 (16.6)	202 (20.6)	28 (6.9)	<0.001
Use of glucose-lowering drugs	91 (6.6)	75 (7.7)	16 (3.9)	0.011
Use of statins	138 (9.9)	103 (10.5)	35 (8.6)	0.279
Use of NSAIDs	97 (7.0)	64 (6.5)	33 (8.1)	0.294
Glucose tolerance status				
NGT	827 (59.5)	552 (56.2)	275 (67.6)	0.002
i-IFG	99 (7.1)	70 (7.1)	29 (7.1)	
i-IGT	160 (11.5	121 (12.3)	39 (9.6)	
IFG–IGT	75 (5.4%)	58 (5.9)	17 (4.2)	
Newly detected T2D	117 (8.4)	89 (9.1)	28 (6.9)	
Previously known T2D	111 (8)	92 (9.4)	19 (4.7)	
Duration of known T2D, y	8 (4–14)	8 (4–14)	7 (5–12)	0.005

Continuous and categorical basic characteristics (covariates) were summarized as median (interquartile range), and counts (percentage), respectively. IFG indicates impaired fasting glucose; i-IFG, isolated impaired fasting glucose; IGT impaired glucose tolerance; i-IGT, isolated impaired glucose tolerance; NGT, normal glucose tolerance; and T2D, type 2 diabetes.

*Difference in continuous and categorical covariates between S4 and S4-FF4 study samples were tested with Kruskal–Wallis rank-sum and Pearson's χ^2 tests, respectively.

robust predictor variables for Rényi4, TP, SDWS, SDSA and CE, respectively (Table 2). The robust proteomics biomarkers include N-terminal pro-B-type natriuretic peptide for Rényi4, tumor necrosis factor–related activation-induced cytokine (TRANCE) for TP, tumor necrosis factor receptor superfamily member 10A for SDWS, N-terminal pro-B-type natriuretic peptide for SDSA and N-terminal pro-B-type natriuretic peptide for CE. The robust covariates were CRP, HbA_{1c}, waist circumference and leukocyte count for Rényi4, waist

Table 2. Robust Predictor Variables of S4 Study Sample

	Rényi4	Abbreviations
1	N-terminal pro-B-type natriuretic peptide	NT-proBNP
2	Tumor necrosis factor receptor superfamily member 10A	TNFRSF10A
3	C-C motif chemokine 23	CCL23
4	Interleukin-6	IL-6
5	Thrombospondin-2	THBS2
6	Insulin-like growth factor-binding protein 1	IGFBP1
7	C-reactive protein	CRP
8	Tumor necrosis factor–related activation-induced cytokine	TRANCE
9	Neurotrophin-3	NT3
10	Peptidoglycan recognition protein 1	PGLYRP1
11	Interleukin-1 receptor-like 2	IL1RL2
12	Hemoglobin A_{1c}	HbA_{1c}
13	Waist circumference	
14	Leukocyte count	
15	Protein α_1-microglobulin/bikunin precursor	AMBP
16	Fibroblast growth factor 21	FGF21
	TP	
1	Tumor necrosis factor–related activation-induced cytokine	TRANCE
2	Low affinity immunoglobulin γ Fc region receptor II-b	IGGFC
3	Lipoprotein lipase	LPL
4	Vascular endothelial growth factor D	VEGFD
5	Interleukin-2 receptor subunit α	IL2RA
6	Tyrosine-protein kinase receptor UFO	AXL
	SDWS	
1	Tumor necrosis factor receptor superfamily member 10A	TNFRSF10A
2	Interleukin-1 receptor-like 2	IL1RL2
3	C-C motif chemokine 23	CCL23
4	Tumor necrosis factor–related activation-induced cytokine	TRANCE
5	Thrombospondin-2	THBS2
6	Spondin-2	SPON2
7	Transforming growth factor alpha	TGFA
8	N-terminal pro-B-type natriuretic peptide	NT-proBNP
9	Interleukin-10 receptor subunit beta	IL10RB
10	Waist circumference	
	SDSA	
1	N-terminal pro-B-type natriuretic peptide	NT-proBNP
2	Thrombospondin-2	THBS2
3	Low affinity immunoglobulin gamma Fc region receptor II-b	IGGFC
4	Receptor for advanced glycosylation end products	RAGE
5	Interleukin-10 receptor subunit β	IL10RB
6	TNF-related activation-induced cytokine	TRANCE
7	Vascular endothelial growth factor D	VEGFD
	CE	
1	N-terminal pro-B-type natriuretic peptide	NT-proBNP
2	Tumor necrosis factor receptor superfamily member 10A	TNFRSF10A
3	Interleukin-6	IL6
4	C-reactive protein	CRP
5	Thrombospondin-2	THBS2
6	Contactin-1	CNTN1
7	C-C motif chemokine 23	CCL23
8	Tumor necrosis factor–related activation-induced cytokine	TRANCE
9	Receptor for advanced glycosylation end products	RAGE
10	Growth/differentiation factor 15	GDF15

CE indicates compression entropy; ényi4, Rényi entropy of the histogram with order (alpha) 4; SDSA, SD of the short-term normal-to-normal interval variability; SDWS, SD of word sequence; and TP, total power of the density spectra.

circumference for SDWS, and CRP for CE. The MUVR-PLS, MUVR-RF, and MUVR-EN regression-specific predictor variables for each CAND–HRV index are provided in Table S3.

No glucose tolerance group was selected by the RF for the 5 HRV indices; as such, there was no complex-full model in model comparison. The comparison of the full and basic models for Rényi4, SDWS, and CE indicated that the full models of Rényi4 (0.83) and CE (0.83) had overall higher performance scores than the basic models of Rényi4 (0.17) and CE (0.17), while the full and basic models of SDWS had equal performance scores of 0.5 (Table S4). Hence, the best-performing models for Rényi4, SDWS, and CE were the full, basic, and full models, respectively. The proteomics biomarkers-only (full) models of TP and SDSA were their best-performing models.

Table 3 summarizes the β and 95% CI of the best-performing models. Key model assumptions, homogeneity of variance (homoscedasticity), normality of residuals, and acceptable multicollinearity (all variance inflation factors were <10) were generally satisfied. Five proteins remained independently associated with 2 indices, cross-sectionally. Specifically, 1-SD higher CCL23 (C-C motif chemokine 23) was associated with 0.10-bit higher Rényi4, while 1-SD higher PGLYRP1 (peptidoglycan recognition protein 1) and FGF21 (fibroblast growth factor 21) were both associated with 0.15-bit lower Rényi4. Further, 1-SD higher TRANCE and GDF15 (growth/differentiation factor 15) were associated with 0.02-AU lower and 0.03-AU lower CE, respectively.

Table 3. Effect Estimates of the "Best"-Performing Models of S4 Study Sample

Regression coefficients (β) (95% CI); *P* value, permutated *P* value	Abbreviations	Rényi4, bit	TP, ms^2	SDWS, AU	SDSA, ms	CE, AU
N-terminal pro-B-type natriuretic peptide	NT-proBNP	0.01 (−0.09 to 0.10); 0.926 to 0.980			1.86 (−0.32 to 4.05); 0.094 to 0.059	0.01 (−0.01 to 0.02); 0.256 to 0.295
Tumor necrosis factor receptor superfamily member 10A	TNFRSF10A	0.044 (−0.05 to 0.14); 0.375 to 0.527				0.001 (−0.01 to 0.02); 0.885 to 0.98
C-C motif chemokine 23	CCL23	0.10* (0.01 to 0.20); 0.039 to 0.047				0.0001 (−0.01 to 0.02); 0.976 to 1
Interleukin-6	IL-6	−0.05 (−0.15 to 0.05); 0.295 to 0.085				−0.002 (−0.017 to 0.014); 0.828 to 1
Thrombospondin-2	THBS2	−0.05 (−0.14 to 0.05); 0.342 to 0.474			−1.26 (−3.41 to 0.89), 0.251 to 0.51	0.0001 (−0.01 to 0.01), 0.980 to 0.961
Insulin-like growth factor-binding protein 1	IGFBP1	0.01 (−0.09 to 0.11); 0.848 to 0.882				
Tumor necrosis factor–related activation-induced cytokine	TRANCE	−0.02 (−0.11 to 0.08); 0.739 to 1	−28.5 (−202.8 to 145.8); 0.748 to 1		−1.04 (−3.06 to 0.99); 0.317 to 0.097	−0.02** (−0.03 to −0.002); 0.024 to 0.039*
Neurotrophin-3	NT3	−0.04 (−0.13 to 0.05); 0.400 to 0.423				
Peptidoglycan recognition protein 1	PGLYRP1	−0.15* (−0.25 to −0.05); 0,004 to 0.002*				
Interleukin-1 receptor-like 2	IL1RL2	−0.04 (−0.14 to 0.05); 0.343 to 1				
Protein α_1-microglobulin/ bikunin precursor	AMBP	0.01 (−0.10 to 0.11); 0.920 to 1				
Fibroblast growth factor 21	FGF21	−0.15* (−0.25 to −0.06); <0.001 to <0.001*				
Low-affinity immunoglobulin γ Fc region receptor II-b	IGGFC		−87.0 (−262.5 to 88.5); 0.331 to 0.403		0.49 (−1.53 to 2.51); 0.637 to 0.563	
Lipoprotein lipase	LPL		20.9 (−154.9 to 196.7); 0.816 to 1			
Vascular endothelial growth factor D	VEGFD		−35.5 (−212.6 to 141.6); 0.694 to 1		1.38 (−0.98 to 3.73); 0.251 to 0.941	
Interleukin-2 receptor subunit α	IL2RA		38.5 (−159.6 to 236.6); 0.703 to 0.941			
Tyrosine-protein kinase receptor UFO	AXL		−160.3 (−357.4 to 36.7); 0.111 to 0.227			
Interleukin-10 receptor subunit β	IL10RB				−0.33 (−2.59 to 1.93); 0.773 to 1	
Receptor for advanced glycosylation end products	RAGE				−0.81 (−3.37 to 1.75); 0.537 to 0.941	0.01 (−0.01 to 0.02); 0.329 to 0.223
Contactin-1	CNTN1					−0.01 (−0.03 to 0.002); 0.086 to 0.062
Growth/differentiation factor 15	GDF15					−0.03 (−0.05 to −0.02); <0.001 to <0.001*

(*Continued*)

Table 3. Continued

Regression coefficients (β) (95% CI); *P* value, permutated *P* value	Abbreviations	Rényi4, bit	TP, ms^2	SDWS, AU	SDSA, ms	CE, AU
C-reactive protein	CRP	−0.06 (−0.17 to 0.04); 0.223 to 1				−0.01 (−0.03 to 0.003); 0.128 to 0.066
Hemoglobin A_{1c}	HbA_{1c}	−0.02 (−0.11 to 0.08), 0.709 to 0.592				
Waist circumference		0.07 (−0.03 to 0.17); 0.192 to 0.189		0.001 (−0.06 to 0.07); 0.786 to 0.51		
Leukocyte count		0.06 (−0.05 to 0.16); 0.308 to 1				

All continuous predictor variables are *Z* score standardized (mean of 0 and SD of 1). Outcome variables are in their original units. CE indicates compression entropy; ényi4, Rényi entropy of the histogram with order (alpha) 4; SDSA, SD of the short-term normal-to-normal interval variability; SDWS, SD of word sequence; and TP, total power of the density spectra.

*Statistically significant estimates.

In addition, the robustness of inference to replacement and impact threshold of a confounding variable estimates of the bias analysis indicated that the association between CCL23 and Rényi4 was the least robust to bias, while the association between GDF15 and CE was the most robust to bias. Details are provided in Data S1. Furthermore, test RMSE indicated that, on average, predictions were off by 0.69 bit for Rényi4, 817.18 ms^2 for TP, 0.49 AU for SDWS, 18.4 ms for SDSA, and 0.11 AU for CE.

The DDA's homoscedasticity and independence tests indicated that for all associations, the putatively correct causal order did not convincingly outperform the reverse causal order (Table S5). These results suggest that the reverse causal order, that is, the influence of these HRV indices on their associated proteomics biomarkers cannot be excluded with certainty.

Association Between Proteomics Biomarkers and HRV Indices in S4-FF4 Study Sample

There were 5 (4 proteomics biomarkers and 1 covariate), 4 (all proteomics biomarkers), 1 (proteomics biomarker), 5 (all proteomics biomarkers) and 8 (all proteomics biomarkers) robust predictor variables for Rényi4, TP, SDWS, SDSA and CE, respectively (Table 4). The robust proteomics biomarkers include adrenomedullin for Rényi4, myoglobin for TP, myoglobin for SDWS, AGRP (agouti-related protein) for SDSA, and CUB domain-containing protein 1 for CE. Triglycerides was the only robust covariate, observed for Rényi4. The MUVR-PLS, MUVR-RF, and MUVR-EN regression-specific predictor variables are provided in Table S6.

No glucose tolerance group was selected by the RF for the five HRV indices hence there was no complex-full model in model comparison. The comparison of Rényi4's robust predictor variables (full) model with its covariates-only (basic) model indicated that the models have equal performance scores of 0.5 (Table S7). Hence, the basic model with only triglycerides was considered as the best-performing model of Rényi4. The proteomics biomarkers-only (full) models of TP, SDWS, SDSA, and CE were their best-performing models.

Table 5 summarizes the β and 95% CI of the best-performing models. Five proteins remained independently associated with 3 indices over time. Specifically, 1-SD higher interleukin-6 receptor subunit α (IL6RA) was associated with 63 ms^2 higher TP, while 1-SD higher macrophage CSF1 (colony-stimulating factor 1) was associated with 57 ms^2 lower TP. Further, 1-SD higher myoglobin was associated with 2.04-ms higher SDSA, while 1-SD higher AGRP was associated with 1.92-ms lower SDSA. Finally, 1-SD higher gastrotropin was associated with 0.04-AU higher CE, while one-SD higher AGRP was associated with 0.03-AU lower CE.

Moreover, the robustness of inference to replacement and impact threshold of a confounding variable estimates of the bias analysis suggested that association between CSF1 and TP was the least robust to bias, while the association between gastrotropin and CE was the most robust to bias. Details are provided in Data S1. Besides, the test RMSE indicated that on average, predictions were off by 0.49 bit, 172 ms^2, 0.43 AU, 6.4 ms, and 0.09 AU for Rényi4, TP, SDWS, SDSA, and CE, respectively.

Collectively, in both study samples, 10 proteomics biomarkers—CCL23, PGLYRP1, FGF21, TRANCE, GDF15, CSF1, IL6RA, AGRP, myoglobin, and gastrotropin—were associated with 4 HRV indices, Rényi4, TP, SDSA, and CE (Table 6).

Table 4. Robust Predictor Variables of S4-FF4 Study Sample

	Rényi4	Abbreviations
1	Adrenomedullin	ADM
2	Myoglobin	MB
3	Triglycerides	
4	C-C motif chemokine 16	CCL16
5	Stem cell factor	SCF
	TP	
1	Myoglobin	MB
2	Protein α_1-microglobulin/bikunin precursor	AMBP
3	Macrophage colony-stimulating factor 1	CSF1
4	Interleukin-6 receptor subunit α	IL6RA
	SDWS	
1	Myoglobin	
	SDSA	
1	Agouti-related protein	AGRP
2	Myoglobin	MB
3	Interleukin-10 receptor subunit β	IL10RB
4	Kidney injury molecule	KIM1
5	Fatty acid-binding protein, intestinal	FABP2
	CE	
1	CUB domain-containing protein 1	CDCP1
2	Angiopoietin-1 receptor	TIE2
3	Gastrotropin	GT
4	Tumor necrosis factor receptor superfamily member 9	TNFRSF9
5	Tumor necrosis factor–related apoptosis-inducing ligand	TRAIL
6	Agouti-related protein	AGRP
7	Serpin A12	SERPINA12
8	Decorin	DCN

CE indicates compression entropy; énYi4, Rényi entropy of the histogram with order (alpha) 4; SDSA, SD of the short-term normal-to-normal interval variability; SDWS, SD of word sequence; and TP, total power of the density spectra.

Independence of Sets of Proteomics Biomarkers, Bivariable Associations of Predictor Variables, and Statistical Power

There was dependency between all 3 sets of proteomics biomarkers (Table S8). Additionally, there were several strong ($|r|\geq 0.7$, $P\leq 0.05$) pairwise correlations between individual proteomics biomarkers as well as between HRV indices of S4 (Table S9). Covariates were moderately associated with the proteomics biomarkers (Tables S9 and S10), while continuous covariates showed fewer associations (Table S9) as compared with categorical covariates (Table S11) with HRV indices of S4. For S4-FF4, individual proteomics biomarkers and HRV indices showed similar magnitude of pairwise correlations as the S4 (Table S12), but fewer covariates were associated with the proteomics biomarkers (Tables S12 and S13) and with HRV indices (Tables S12 and S14). All these results confirm the appropriateness of the a priori multivariable modeling approach with variable selection that adequately accounts for multicollinearity and dependency. Moreover, the statistical power of the generalized linear model of each of training, validation, and testing data sets of S4 was ≈100%. The S4-FF4 training and testing data sets had 63% and 35% power, respectively.

The robust predictor variables of individual power components, ultra-low frequency, very low frequency, low frequency, and high frequency were proteomics biomarkers, except for high frequency in the S4-FF4 (Table S15). No proteomics biomarker was significantly associated with their respective power components in the holdout data sets (Table S16). There is a consistent absence of association of proteomics biomarkers with individual power and TP cross-sectionally in S4, while 2 proteomics biomarkers were significantly associated with TP and none with individual power in S4-FF4. All Supplemental Materials are accessible at https://figshare.com/s/12c7ae9a7bd27a85f8a2.

DISCUSSION

In this population-based epidemiological study of German older adults, we uncovered 10 novel proteomics biomarkers—CCL23, PGLYRP1, FGF21, TRANCE, GDF15, CSF1, IL6RA, AGRP, myoglobin, and gastrotropin—that were associated with 4 HRV indices: Rényi4, TP, SDSA, and CE. Our findings are intriguing in the light of the dual roles of several inflammatory biomarkers,[34] the antagonistic but dynamic balance of sympathetic and parasympathetic activities on HRV,[35] higher HRV generally deemed to be health preserving,[36] and severely diminished HRV reflecting CAND.[37]

Association of CCL23, PGLYRP1, and FGF21 With Rényi4

CCL23 was positively associated with Rényi4, while PGLYRP1 and FGF21 were negatively associated with Rényi4. Rényi4 is a measure of the complexity, diversity, uncertainty, or randomness of the beat-to-beat intervals[9,38] and evenly captures linear and nonlinear variability.[9] Rényi4 is generally higher in healthy individuals as compared with those with cardiac abnormalities.[38] CCL23, a chemokine expressed by macrophages in the lungs, liver, and pancreas stimulates the production of proinflammatory cytokines and adhesion molecules.[39] It is associated with neuroinflammation[39] and related to chronic diseases with inflammatory components such as rheumatoid arthritis,[40] systemic sclerosis,[41] ischemic stroke,[42] coronary

Table 5. Effect Estimates of the "Best"-Performing Models of S4-FF4 Study Sample

Regression coefficients (β) (95% CI); *P* value, permutated *P* value	Abbreviations	Rényi4, bit	TP, ms^2	SDWS, AU	SDSA, ms	CE, AU
Myoglobin	MB		42.9 (−3.2 to 89.0); 0.068 to 0.068	0.05 (−0.03 to 0.14); 0.222 to 0.065	2.04 (0.55 to 3.53); 0.008 to <0.001*	
Protein α_1-microglobulin/bikunin precursor	AMBP		−34.2 (−86.0 to 17.6); 0.194 to 0.194			
Macrophage colony-stimulating factor 1	CSF1		−57.0 (−107.0 to −6.9); 0.026 to <0.001			
Interleukin-6 receptor subunit α	IL6RA		63.0 (20.0 to 105.9); 0.004 to <0.001*			
Agouti-related protein	AGRP				−1.92 (−3.45 to −0.39); 0.014 to <0.001*	−0.03 (−0.05 to −0.01); 0.008 to <0.001*
Interleukin-10 receptor subunit β	IL10RB				−1.40 (−3.01 to 0.22); 0.089 to 0.089	
Kidney injury molecule	KIM1				−1.01 (−2.57 to 0.41); 0.153 to 0.153	
Fatty acid-binding protein, intestinal	FABP2				1.50 (−0.03 to 3.04); 0.055 to 0.055	
CUB domain-containing protein 1	CDCP1					0.003 (−0.02 to 0.02); 0.772 to 0.603
Angiopoietin-1 receptor	TIE2					−0.01 (−0.03 to 0.01); 0.337 to 0.064
Gastrotropin	GT					0.04 (0.02 to 0.06); <0.001 to <0.001*
Tumor necrosis factor receptor superfamily member 9	TNFRSF9					−0.01 (−0.04 to 0.01); 0.192 to 0.192
Tumor necrosis factor–related apoptosis-inducing ligand	TRAIL					−0.002 (−0.02 to 0.02); 0.818 to 0.818
Serpin A12	SERPINA12					0.01 (−0.01 to 0.03); 0.470 to 0.470
Decorin	DCN					−0.01 (−0.03 to 0.02); 0.672 to 0.672
Triglycerides		−0.13 (−0.23 to −0.02); 0.020 to <0.001				

CE indicates compression entropy; ényi4, Rényi entropy of the histogram with order (alpha) 4; SDSA, SD of the short-term normal-to-normal interval variability; SDWS, SD of word sequence; and TP, total power of the density spectra.

*Statistically significant estimates. All continuous predictor variables are *Z* score standardized (mean of 0 and SD of 1. Outcome variables are in their original units.

artery calcium,[43] atherosclerosis,[44] and Alzheimer disease.[39] CCL23 plays a role in angiogenesis,[45] which is part of vascular remodeling. This is a potential explanation for its association with Rényi4. PGLYRP1 is primarily expressed in leukocytes, providing antimicrobial and proinflammatory functions.[46] Its higher blood level is linked to increased CVD risk.[47] FGF2 is synthesized in the liver, pancreas, adipose tissue, and skeletal muscle,[48] as well as in cardiomyocytes.[49] It is involved in the regulation of metabolism and anti-inflammatory processes.[50] It plays a protective role in diabetic cardiomyopathy and prevents cardiac damage.[51] The mechanisms underlying its cardioprotective role are regulation of adipocyte adiponectin production and suppression of hepatic expression of the transcription factor sterol regulatory element-binding protein-2.[52] However, FGF21 is also associated with increased risk of secondary CVD,[52] which suggests that its negative association with Rényi4 is plausible. The association between FGF21 and Rényi4 may also be a reflection of the potential link between hepatic steatosis and early development of CAND.[5]

Association of TRANCE, GDF15, AGRP, and Gastrotropin With CE

TRANCE and GDF15 were negatively associated with CE, while higher baseline AGRP and gastrotropin

Table 6. Overall Results of the Proteomics Biomarkers Significantly Associated With Cardiac Autonomic Nervous Dysfunction–Heart Rate Variability Indices

	Proteomics biomarkers	Abbreviations	S4 study sample	S4-FF4 study sample	CAND-HRV indices
1	C-C motif chemokine 23	CCL23	+		Rényi4
2	Peptidoglycan recognition protein 1	PGLYRP1	–		Rényi4
3	Fibroblast growth factor 21	FGF21	–		Rényi4
4	Tumor necrosis factor–related activation-induced cytokine	TRANCE	–		CE
5	Growth/differentiation factor 15	GDF15	–		CE
6	Interleukin-6 receptor subunit alpha	IL6RA		+	TP
7	Macrophage colony-stimulating factor 1	CSF1		–	TP
8	Myoglobin	MB		+	SDSA
9	Agouti-related protein	AGRP		–	SDSA
9	Agouti-related protein	AGRP		–	CE
10	Gastrotropin	GT		+	CE

CAND-HRV indicates cardiac autonomic nervous dysfunction-heart rate variability; CE, compression entropy. Rényi4, Rényi entropy of the histogram with order (α) 4; SDSA, SD of the short-term normal-to-normal interval variability; and TP, total power of the density spectra. +=Positive association; –=Negative association.

were associated with decrease and increase in CE over time, respectively. CE is also a marker of complexity, but more sensitive to nonlinear than linear variability.[9] It generally indicates parasympathetic (vagal) modulation.[53] This suggests that TRANCE, GDF15, and AGRP may be linked with decreased vagal activity, while gastrotropin may be linked with increased vagal activity. TRANCE is expressed by osteoblasts and fibroblasts, activated T cells, subcapsular sinus macrophages, metallophilic macrophages, and certain myeloma.[54] It plays a role in endothelial cell activation, which is pivotal to angiogenesis and proinflammatory processes.[55] Higher serum TRANCE is associated with the Charcot foot, a neuropathic arthropathy,[56] closely linked to preceding neuropathy.[57] Similarly, GDF15 exhibits proinflammatory and anti-inflammatory properties.[58] It is associated with diabetic neuropathy, specifically showing direct and inverse associations with longer sensory and motor nerve latencies and slower nerve conduction velocity, respectively.[59] AGRP is a neuropeptide synthesized by the brain's AGRP/neuropeptide Y neurons, regulating glucose sensing and metabolism.[60,61] AGRP neurons are highly active during hunger, promoting robust feeding behavior.[62] Besides, they mediate the effects of leptin on autonomic nerve activity[63] and the mechanistic relationship between the vagal afferent pathway and the central nervous system.[64] Gastrotropin is one of the fatty acid–binding proteins.[65] It is most abundant in the ileum and transports bile acids,[65] regulating lipid and glucose metabolism.[66] Recent epidemiological investigations reported that gastrotropin is directly associated with CAD[67] but inversely associated with the risk of CVD.[68]

Association of CSF1 and IL6RA With TP

Higher baseline CSF1 and IL6RA were associated with decrease and increase in TP over time, respectively. Sympathetic activation and its resulting tachycardia are usually accompanied by a marked reduction in TP, while the reverse occurs during vagal activation.[69] These findings suggest that CSF1 and IL6RA are associated with higher and lower sympathetic activity, respectively. CSF-1, expressed in the brain[70] and enteric neurons,[71] is one of the most common proinflammatory cytokines involved in somatosensory and autonomic neuronal regulatory processes.[71] It mediates microglial and macrophage signaling in the generation of neuropathic pain, which occurs after nerve injury.[72] It is responsible for various inflammatory disorders.[73] In fact, its genetically predicted higher levels are linked to higher risk of coronary artery disease.[74] IL6RA is a transmembrane protein expressed on hepatocytes,[75] leukocytes,[75] adipocytes,[76] myocytes,[77] and right atrium.[78] Most of the proinflammatory roles of interleukin-6 are attributed to its binding to soluble IL6RA.[75] CSF-1 and some interleukins have overlapping binding sites.[70] Hence, our observed association of CSF-1 and IL6RA with TP may suggest their concerted cardiac autonomic regulatory action. Surprisingly, these proteins were not associated with individual power components, suggesting that these indices may be less reflective of the cardiac impact of these proteins.

Association of Myoglobin and AGRP With SDSA

Higher baseline myoglobin and AGRP were associated with an increase and a decrease in SDSA over time,

respectively. SDSA is a measure of both parasympathetic and sympathetic activity.[78] These findings suggest that myoglobin and AGRP may be necessary for maintaining a dynamic balance between cardiac parasympathetic and sympathetic modulations. Myoglobin is primarily expressed in skeletal and cardiac muscles.[79] It protects the cardiovascular system through storage and facilitation of dioxygen diffusion.[80] The production and role of AGRP has been discussed with respect to CE.

Influence of Glucose Tolerance Status on the Relationship of Proteomics Biomarkers With HRV Indices

Contrary to our expectations, none of the glucose tolerance groups relative to NGT seemed to have an important influence on the relationship between any of these biomarkers and their respective HRV indices. This is in spite of the glucose tolerance status having bivariable associations with FGF21 and GDF15 (Table S8) and with CSF1, myoglobin, and AGRP in the S4-FF4 (Table S11). Moreover, as compared with NGT, isolated impaired fasting glucose, known T2D, or newly detected T2D were associated with indices in 1 or 2 variable selection models of S4 (Table S1) and S4-FF4 (Table S4). However, none was a robust predictor for any index. These findings suggest that in the presence of other proteomics biomarkers and risk factors, glucose tolerance status is unlikely to exert a substantial influence on the association between these 10 proteomics biomarkers and HRV indices. This underscores the need for a nuanced understanding of role of glucose tolerance status, especially T2D, in the relationship between these current biomarkers and CAND.

Previously Reported Biomarkers and Risk Factors for CAND

CRP,[13,15] interleukin-6,[15] interleukin-18,[16] interleukin-1 receptor antagonist,[15] and adiponectin[14,16] are linked to CAND. Additionally, a review predating these studies indicated that parasympathetic nervous system tone as inferred from HRV is inversely related to CRP and interleukin-6.[81] Reassuringly, across the variable selection models of both study samples, we observed the association of these biomarkers with at least 1 HRV index. However, in the S4, only CRP and interleukin-6 were robust, as both were associated with Rényi4 and CE cross-sectionally. However, none was validated. In contrast, none of these previously reported biomarkers were robust in the longitudinal S4-FF4 analysis. This suggests that, despite their widespread importance in pathophysiological processes, CRP and interleukin-6 are unlikely to provide added value beyond these 10 novel proteomics biomarkers for Rényi4, TP, SDWS, SDSA, and CE.

Similarly, we observed previously reported risk factors of CAND such as age, sex, obesity, smoking, blood pressure, dyslipidemia, and dysglycemia in at least 1 variable selection model. However, they were simply not robust as compared with HbA_{1c}, waist circumference, or triglycerides. The associations between HbA_{1c} and Rényi4 and between waist circumference and SDWS were not validated. HbA_{1c} was associated with some HRV indices in a study that did not include Rényi4 and SDWS.[82] Interestingly, triglycerides were validated in our S4-FF4 study sample, showing a negative association with Rényi4. This is in support of inverse association of triglycerides with prevalent cardiac autonomic neuropathy.[82]

Strengths and Limitations

One of the strengths of this study is that it is the largest study exploring proteomic biomarkers of CAND. Additionally, this targeted profiling of proteomics biomarkers includes known proteins with documented biological roles. This helps place our findings in proper context. The multivariable selection models with repeated cross-validation ensures the precision and reliability of predictor variable selection. Further, the selection of variables across 3 methods reduces bias inherent in any method. The adequate inclusion of the glucose tolerance group, as a stratifying variable for data splitting and in the multivariable selection helps avoid omitted variable bias. Given the complicated nature of statistical power analysis in mixed models,[83] all our power estimates assumed single measurement, but as repeated measurements typically have higher power than single measurement,[84] the S4-FF4 data sets are unlikely to be underpowered. Hence, we efficiently tested our hypotheses and obtained reliable β. Moreover, the inferential estimates of the predictor variables, which were obtained from distinct data sets help to reduce the risk of erroneous results and inflated performance metrics. Rather than merely acknowledging the limitations of the cross-sectional S4, our DDA ensures that we are not overly confident in the findings of the a priori causal flow. Further, the bias analyses reinforced the reliability of our inferences. The longitudinal S4-FF4 helps control for unobserved time-specific heterogeneity. Although the S4-FF4 has the temporal ordering advantage over the S4, the fact that our final protein–indices associations are distinct suggests that these proteins are likely to have clinical translational relevance. Therefore, joint investigation of both study samples is also a merit of this work. Finally, our study accounted for several cardiometabolic risk factors as well as commonly assessed inflammatory biomarkers. This suggests that the impact of these

novel biomarkers on HRV indices is likely independent of these factors.

The limitations of our study include its observational nature, hence we cannot draw a definite conclusion on causal relationships. Overall, we found small to moderate effect estimates in all associations so that the clinical relevance of our findings remains to be determined. Although the most efficient method to ensure the reliability and predictive ability of these identified proteomics biomarkers would have been through external replication in a purely independent cohort, the reliability of our findings was achieved using the widely accepted holdout approach. Although within-study validation indicates the reliability of proteomics biomarkers–HRV indices association, nonetheless it was surprising that no proteomics biomarkers–HRV indices association overlapped between studies in the final S4 validation and S4-FF4 testing data sets. However, 1 association, interleukin-10 receptor subunit β with SDSA, overlapped between the S4 and S4-FF4 training data sets. This association was not validated in both S4 validation and S4-FF4 testing data sets, suggesting consistency. It is possible that bivariable selection models and a single multivariable selection model, which are likely more prone to bias as compared with the current rigorous and robust multivariable selection approach, might have provided more overlapping associations to be subsequently validated. Despite combinations of our covariates being likely reasonable surrogates for uncontrolled and residual confounding, these issues might still have an impact on our findings. These confounders may include HRV-influencing health conditions such as depression.[85] Residual confounding may also arise from lack of flexible modeling of covariates. Besides, we cannot completely exclude untoward consequences of excluding some individuals on the basis of the absence of the exposure, outcome, and glucose tolerance status. However, the low proportion of the excluded suggests that its impact on our findings is likely trivial. As expected in most cohorts of older adults, the S4-FF4 study sample was comparatively smaller than the S4 study sample due to the attrition of the study participants at follow-up who were already older adults at baseline. Additionally, these current HRV indices were obtained from short-term, 5-minute measurement. Findings for indices from long-term measurements such as 24-hour might be different. The binary outcome (CAND or no CAND) could have been of added value to the individual HRV indices. However, this was not possible for logistic reasons. Actually, the biological interpretation of biomarkers associated with binary outcome would still be heavily hinged on the clinical relevance of the individual HRV indices. Another limitation is that prospectively linking baseline proteins to indices assessed at baseline and at 14 years later in S4-FF4 assumes that circulating levels of proteins are stable over this period. This assumption may not hold for all proteins. Multiple targeted proteomics profiling as well as additional assessments of HRV indices will increase the validity of these findings. While the complexity of untargeted proteomics profiling may be daunting, this approach has merits that warrant its consideration in future studies with larger sample size. Although our held-out data sets are well powered, it is possible that causal direction did not adequately manifest itself in the association between proteins and HRV indices, which the current DDA tests rely on. Future studies should consider pathway enrichment analysis of these proteins and integrate them into a broader pathophysiological model as well as in risk stratification framework for CAND. Validation of these novel proteins using highly sensitive and specific methods such as ELISA will ensure the robustness of our findings.

In conclusion, this population-based epidemiological study adds to the emerging knowledge on inflammatory and cardiovascular biomarkers of CAND. We observed that independent of glucose tolerance status and other risk factors, plasma levels of 10 novel proteomics biomarkers, CCL23, PGLYRP1, FGF21, TRANCE, GDF15, CSF1, IL6RA, AGRP, myoglobin, and gastrotropin, are related to 4 HRV indices. These biomarkers reflect aspects of the pathophysiology of CAND, which have not been previously reported. Certainly, the clinical manifestation of CAND is likely a consequence of multiple risk factors and biomarkers intricately interacting together. Nonetheless, these novel biomarkers may be valuable in understanding and dissecting some aspects of manifestation of CAND. A deeper understanding of the roles of these proteins under various conditions could advance the therapeutic strategies for CAND.

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Received March 11, 2025; accepted July 21, 2025.

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Acknowledgments

The authors thank all participants for their long-term commitment to the KORA study, the staff for data collection and research data management and the members of the KORA Study Group (https://www.helmholtz-munich.de/en/epi/cohort/kora) who are responsible for the design and conduct of the study. K.O., C.H., B.T., and D.Z. designed the research; S.M.H., A.P., M.H., C.M., A.P., B.T., and A.V. acquired the data; A.S., G.B., M.R., W.R., M. F.S., and S.K. provided essential materials; K.O. developed the statistical analytical plan, performed all statistical analyses, interpreted the results, and wrote the first draft of the manuscript; K.O. and C.H. have primary responsibility for the final content; and all authors read and approved the final manuscript.

Sources of Funding

The German Diabetes Center is funded by the German Federal Ministry of Health (Berlin, Germany) and the Ministry of Culture and Science of the state North Rhine–Westphalia (Düsseldorf, Germany) and receives additional funding from the German Federal Ministry of Education and Research through the German Center for Diabetes Research (DZD e.V.). The KORA study was initiated and financed by the Helmholtz Zentrum München–German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research and by the State of Bavaria. Data collection in the KORA study is done in cooperation with the University Hospital of Augsburg.

Disclosures

None.

Supplemental Material

Data S1
Tables S1–S16
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